*NUCLEAR MAGNETIC RESONANCE STUDIES OF HEMOGLOBINS, III. EVIDENCE FOR THE NONEQUIVALENCE OF α- AND β-CHAINS IN AZIDE DERIVATIVES OF METHEMOGLOBINS**

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Abstract.—Nuclear magnetic resonance spectroscopy (100-MHz proton) was used to study the low-spin ($S = 1/2$) azide derivatives of human adult ($\alpha_2\beta_2$), human fetal ($\alpha_2\gamma_2$), Zürich ($\alpha_2\beta_2^{63\ \text{His} \rightarrow \text{Arg}}$), and horse ($\alpha_2'\beta_2'$) methemoglobins, as well as whale metmyoglobin in 0.1 M deuterated phosphate at pD 7 and at 31°C. The experimental results indicate that the azide-bound heme groups of the α- and β-chains in human adult methemoglobin and of the α- and γ-chains in fetal methemoglobin are not equivalent. The affinity of the β- or γ-chain for azide ion appears larger than that of the α-chain. The nuclar magnetic resonance spectrum of hemoglobin Zürich shows that the environment of the azide-heme complex in the abnormal β-chain is altered by the substitution of arginine for histidine in the β-63 position, while the α-heme environment remains unaffected.

Introduction.—The chief physiologic function of hemoglobin is to transport molecular oxygen from lung to tissues by virtue of its ability to combine reversibly with oxygen. Hemoglobin is a protein molecule consisting of four subunits (normally two α-chains and two β-chains). Each of the four subunits has a heme group, and these heme groups are the "active centers" of hemoglobin. Many of the functional properties of hemoglobin are highly sensitive to the quarternary structure of the molecule.[1, 2] Perutz and Lehmann[3] recently pointed out that although these properties are, in general, insensitive to replacements of most amino acid residues on its surface, they may be profoundly altered by relatively small changes in internal nonpolar contacts, such as those near the heme and those contacts between the subunits. Hence, a knowledge of the reactivity, as well as the equivalence or nonequivalence of the heme groups in the individual chains of hemoglobins toward ligands, and of the detailed environment of these groups will be of value in our understanding of structural-functional relationship in hemoglobins.

Because the nuclear magnetic resonance (NMR) shifts of the protons in paramagnetic hemin derivatives are shifted by electron hyperfine interactions[4, 5] to regions of the spectrum outside the range for diamagnetic compounds, it is possible to probe the heme environment of hemoproteins by NMR spectroscopy.[6–9] Proton NMR data (reported by us[6, 7] and others[8–10]) on various high- and low-spin forms of metmyoglobin and methemoglobins reveal that the heme proton resonance shifts depend on the heme environment in these proteins. In a recent communication, we showed NMR spectra for three cyanomethemoglobins which were interpreted in terms of interactions between some heme methyl groups and specific amino acid residues which are in close proximity to the heme group, namely, residues 70 and 71 in β- and γ-chains.[7] This interpretation

was made with the knowledge of the primary structure of these proteins and the recent X-ray data for horse oxyhemoglobin at 2.8 Å resolution by Perutz *et al.*[2]

In this paper, we wish to report the 100-MHz proton NMR spectra of the azide ion derivatives of human adult [Hb A ($\alpha_2\beta_2$)], human fetal [Hb F ($\alpha_2\gamma_2$)], Zürich [Hb Zürich ($\alpha_2\beta_2^{63\text{His}\rightarrow\text{Arg}}$)], and horse [Hb ($\alpha_2'\beta_2'$)] methemoglobins as well as of whale metmyoglobin. Hemoglobin Zürich is an abnormal hemoglobin in which the distal histidine residue at β-63 position has been replaced by an arginine residue.[11, 12] Heterozygous carriers have mild hemolytic anemia, which may show fulminant exacerbation if the patient receives sulfonamides.[3, 13] In addition to substantiating our early interpretation of the spectra obtained with the cyano derivatives of MetHb A, MetHb F, and horse MetHb,[7] the present results indicate that for the azide derivatives, the heme groups in the two different chains are nonequivalent and that the affinity or reactivity of the azide ion for the β-chain heme in Hb A or the γ-chain in Hb F is greater than that of the α-chain heme. These conclusions are strengthened by the observation of the NMR spectrum of an abnormal hemoglobin, i.e., Hb Zürich. These findings are also in agreement with hemoglobin azide ion kinetic and equilibrium binding studies by us[14] and by other investigators.[15, 16]

Experimental.—Fresh human blood samples were obtained from the local blood bank. Adult hemoglobin solutions were prepared by the usual procedure. The red cells were lysed either by toluene or by distilled water. HbO_2 was oxidized by two- to sixfold excess of potassium ferricyanide. MetHb solution was concentrated to about 25% (gm/100 ml) by the ultrafiltration cell with XM-10 Diaflo membrane manufactured by the Amicon Corporation. MetHb solution was finally dialyzed against D_2O (purchased from Bio-Rad) at 4°C to replace H_2O. $MetHbN_3$ was prepared by adding the appropriate amount of 1 *M* deuterated phosphate in 0.5 *M* KN_3 to give a final pD 6.8 to 7.2. Horse hemoglobin solution for NMR study was prepared in the same way as that of human adult hemoglobin.

Human fetal hemoglobin solutions were prepared from fresh cord blood obtained from the Magee Women's Hospital. Cord blood hemoglobin was prepared in the same manner as that of the human adult hemoglobin. Hb F fraction was eluted from the cord blood hemoglobin by the procedure of Zade-Oppen.[17] The fetal $MetHbN_3$ for NMR study was prepared the same as that of adult $MetHbN_3$ described above.

Hemoglobin Zürich solutions were prepared from the blood samples drawn from two carriers of this variant,[13] with EDTA as an anticoagulant. Red cells were washed three times, diluted with 2 vol of distilled water, and shaken with approximately 0.1 vol of carbon tetrachloride. Approximately 500 mg of hemoglobin was applied to a $1 \times 8''$ column of DEAE-Sephadex, and the components were separated according to the procedure of Dozy *et al.*[18] After chromatography, desired portions of the eluate were concentrated by ultrafiltration through Visking dislysis membranes. Concentrated fractions were stored at −70°C until studied further. The azide form of the MetHb Zürich was prepared by the addition of fivefold excess of $K_3Fe(CN)_6$ in the presence of 0.01 *M* KN_3 so as to avoid protein precipitation after oxidation to the met-form. The excess ferri- and ferro-cyanide was removed by passing it through a column of G-25 Sephadex (Pharmacia) in 0.1 *M* Na_2CO_3 and in 0.01 *M* KN_3 at pH 8.5. The pH was then converted to about 6.2 by passage of the Hb Zürich solution through a G-25 Sephadex column in 0.05 *M* phosphate and in 0.01 *M* KN_3 at pH 6. $MetHbN_3$ Zürich solution was then concentrated and dialyzed against D_2O.

Myoglobin (whale skeletal muscle, A grade) was obtained from Calbiochem. The $MetMbN_3$ solution for NMR study was prepared in the same manner as that for adult hemoglobin. The NMR spectrum of MetMbCN prepared from this material is indis-

tinguishable from the spectrum of MetMbCN sample prepared and kindly supplied to us by Drs. F. N. Gurd and T. E. Hugli.

All chemicals in this study were commercially available from major suppliers and were used without further purification. The pD of the hemoglobin solutions was obtained from the direct pH reading plus 0.4 unit from a radiometer pH meter (model 26) and a Beckman model 39036 frit junction combination electrode.

The 100-MHz proton NMR spectra were obtained from a Varian HA-100 spectrometer. The spectrometer was operated in the HR-mode, and audio side bands of the residual HDO peak were used to calibrate the spectra. The ambient temperature of the probe was 31°C. The chemical shifts are expressed in parts per million (ppm) from HDO where shifts to low field are assigned negative values.

The hemoglobin concentration for all samples used was varied from 4.5×10^{-3} M to 1.2×10^{-2} M (in terms of heme) in 0.1 M deuterated buffer and a given concentration of N_3^- at pD 6.8 to 7.2. The hemoglobin concentration was determined spectrophotometrically by means of a Zeiss PMQ II spectrophotometer using the molar extinction for the cyanomethemoglobin[19] as $\epsilon = 11.0 \times 10^3$ at 540 mμ and that for the methemoglobin azide[14] as $\epsilon = 11.0 \times 10^3$ at 547 mμ.

Results.—A portion of the proton NMR spectra (−30 to −8 ppm from HDO) for the azide forms of human adult, human fetal, and Zürich methemoglobins, as well as that of metmyoglobin, in 0.1 M deuterated phosphate and in 0.05 M KN_3 at pD $\sim$ 6.9 are shown in Figure 1. The prominent lines from −23 to −15 ppm from HDO in these spectra are assigned to some of the heme methyls on the basis of relative line intensities and comparison with the NMR spectra of $MetMbN_3$ and MetHbCN[7, 9] recorded under similar experimental conditions. Above −12 ppm intense, low-field wings of the aromatic residue proton resonances of the protein moiety prevent accurate assessment of the line intensities and possibly obscure additional heme proton resonances. No resonances were observed in the region −50 to −30 ppm from HDO. In the human adult $MetHbN_3$ spectrum, the relative intensities of the lines at −22.4, −21.3, −16.5, and −15.7 (labeled A, B, C, and D, respectively) are approximately 1:1:1:1. For human fetal $MetHbN_3$, the relative intensities of the lines at A, B, and C ppm are approximately 1:1:2. The NMR spectrum of horse $MetHbN_3$ is identical to that of fetal $MetHbN_3$ in this region. In the case of Zürich $MetHbN_3$, the NMR spectrum is very different from that of the normal adult $MetHbN_3$; the relative intensities of the lines at A and C are about 1:1. While it is essentially impossible to measure the absolute intensities of these lines, it was found that in protein solutions with equal concentration of heme, the intensities (i.e., total area under the resonance line) of the heme methyl resonances in $MetMbN_3$ (or MetMbCN[20]) spectra were approximately twice the intensities of lines A, B, C, or D in the human adult $MetHbN_3$ spectrum. It was also found that in the spectra of solutions containing less than saturating amounts of N_3^-, the intensities of lines A and C decreased relative to the intensities of lines B and D (Fig. 2). Similar results were obtained for the lowest field of lines in the fetal $MetHbN_3$ spectrum (Fig. 2).

Discussion.—It has generally been assumed that the heme groups in the two subunits of normal hemoglobins are equivalent and cannot be distinguished chemically or spectroscopically. However, this interpretation for azide methemoglobins does not appear to be consistent either with the data we report here or

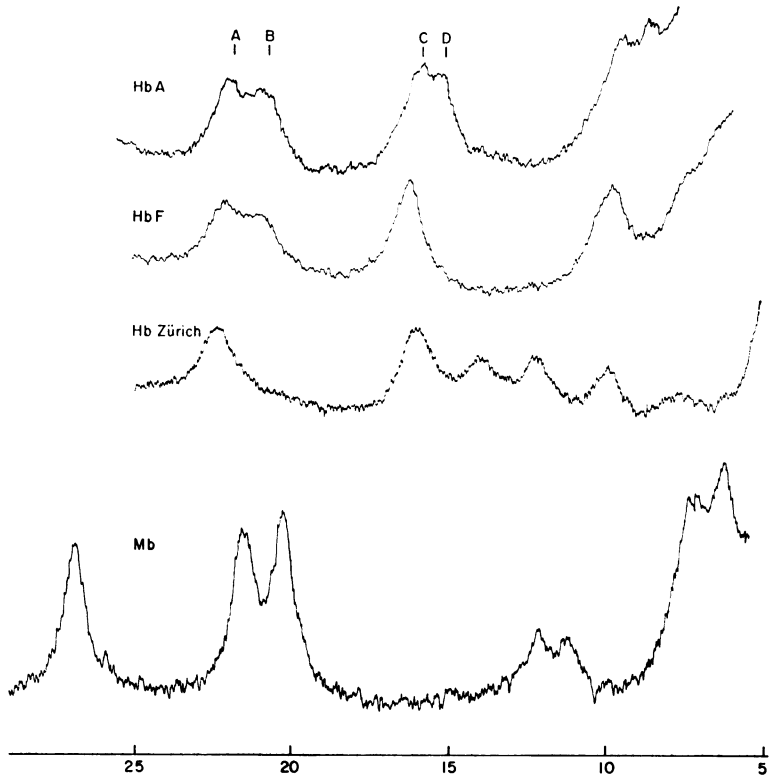

FIG. 1.—100-MHz proton NMR spectra of human adult (Hb A), human fetal (Hb F), and Zürich (Hb Zürich) methemoglobin azide derivatives and metmyoglobin azide (Mb) in the range −28 to −5 ppm from HDO at pD ~7 and at 31°.

with recent kinetic and equilibrium data for the binding of N_3^- to methemoglobins.[14, 15]

The azide ion titration experiment (Fig. 2) which shows that lines *A* and *C* decrease in intensity relative to lines *B* and *D* indicates that lines *A* and *C* can be assigned to a heme group whose binding with azide ion is less than the heme group associated with lines *B* and *D*. Furthermore, the approximate twofold difference in intensity between the heme methyl resonances of $MetMbN_3$ and the lines *A*, *B*, *C*, or *D* implies that any one of these lines belongs to half the total number of hemes in the hemoglobin molecule. The conclusion is that for human adult $MetHbN_3$, the heme groups of the α- and β-chains are not equivalent; this nonequivalence is reflected in both the NMR spectra and the reactivity of azide ion toward methemoglobins.[14, 15]

A number of recent kinetic and equilibrium binding experiments of azide ion to isolated α- and β-chains,[15] intact human adult,[14, 15] human fetal,[14] as well as some abnormal variants[14, 16] of methemoglobin, also agree with our finding that the heme groups in the α- and β-chains or in the α- and γ-chains are not equiva-

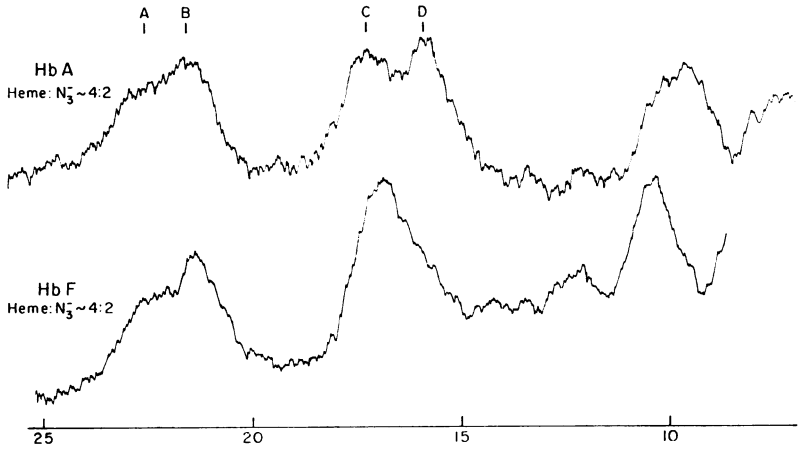

FIG. 2.—100-MHz proton spectra of human adult and human fetal methemoglobin solutions in which the molar ratio of heme to azide ion is approximately 4:2 at pD ~7. Scale is in ppm downfield from HDO at 31°.

lent with respect to azide ion binding.[14-16] The kinetic experiments also suggest that the β-hemes and γ-hemes are more reactive than the α-hemes.[14, 15] These same conclusions can be drawn from the NMR experiment if the fetal $MetHbN_3$ and Zürich $MetHbN_3$ spectra are compared with that of adult $MetHbN_3$.

The amino acid sequence of the γ-chain of human fetal hemoglobin differs from the adult β-chain sequence in 39 places.[21] However, recent X-ray analysis of horse HbO_2[2] and the amino acid sequences of human β- and γ-chains[21] show that only two of these 39 amino acid replacements are within 4 Å of the heme group. In particular, replacement of the human β-chain alanine in residue 70 by a serine in the γ-70 position can introduce a new contact between the serine oxygen and the methyl group in pyrrole ring IV.[7] Our earlier work suggests that this perturbation causes an observable shift in the proton resonance of this methyl group on the γ-chain heme in fetal MetHbCN.[7] This same perturbation appears in the fetal $MetHbN_3$ spectrum, but the resonance shift is presumably in the opposite direction. The resonance lines at *A* and *B* of fetal $MetHbN_3$ are identical with those in adult $MetHbN_3$, the intensity at *C* has doubled, and no line appears at *D*. These same features are also present in the spectra of horse $MetHbN_3$, a hemoglobin which also has a serine residue in the horse β-70 position.[21] Because the titration experiments show that the lines at *A* and *C* belong to the same heme group, and the same decrease in relative intensity is found between the pair of lines at −22.4 and −21.3 ppm in fetal methemoglobin titrations with azide ion, the conclusion is that the lines at −22.4 and −16.5 ppm belong to the α-chain heme and this heme group binds azide ion less strongly than the β- or γ-heme. It is also inferred that the lines *B* and *D* belong to the β-heme.

These conclusions are given additional weight by the result of the proton NMR spectrum of the azide form of MetHb Zürich.[22] Qualitatively, the NMR spec-

trum of this hemoglobin is quite different from that of Hb A.[23] This is to be expected because the abnormality in Hb Zürich is due to the substitution of the distal histidine residue at the β-63 position by a bulky arginine residue. This group can alter the nature of the heme-azide interaction and, therefore, the NMR spectrum of the β-chain heme protons. Perutz and Lehmann[3] have pointed out that this arginine residue in Hb Zürich cannot be accommodated in the heme pocket but protrudes at the surface, leaving a large cavity at the ligand site of the iron atom. In any case, our present NMR results indicate that the heme environment in the abnormal β-chain is different from that of the normal one. Quantitatively, the lines at -22.4 and -16.5 ppm from HDO are essentially the same as those for adult and fetal $MetHbN_3$. This is consistent with the assignment of these lines to α-heme protons. The absence of the lines at -21.3 and -15.7 ppm is equally consistent with the assignments for the adult $MetHbN_3$ spectrum and reflects the altered environment of the β-heme-azide complex in Hb Zürich. Another implication of these results is that the abnormal β chain does not appear to affect the heme proton resonances of the α-chain (i.e., at least the methyl resonances at -22.4 and -16.5 ppm).

By combining the information obtained from our NMR results and that from other physicochemical techniques, we hope to have a better understanding of the structural-function relationship of human hemoglobins.

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